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Oligonucleotides as Probes for Studying Polymerization Reactions in Dilute Aqueous Solution: II. Polycondensations

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Abstract. We have prepared a [^{32}P]-labeled oligonucleotide probe carrying a ureido (-NH-CO-NH_2) function at its 3′-terminus. This labeled oligomer was used to study polycondensations of urea and formaldehyde and of various phenols and formaldehyde in aqueous solution. The formation of formaldehyde copolymers attached to the amido-function of the probe was monitored by gel electrophoresis. Our results are generally in agreement with those obtained using conventional techniques. Our method is suitable for monitoring potentially prebiotic polycondensation reactions involving formaldehyde.

Key words: Urea-formaldehyde polymers — Phenol-formaldehyde polymers — Oligonucleotide-based initiators — Electrophoretic analysis

Introduction

Recently we described a method in which [^{32}P]-labeled oligonucleotides are used as probes for studying polymerization reactions in dilute aqueous solution (Kolb and Orgel 1994; Zieboll and Orgel 1994). This method has been applied to the polymerization reactions of amino acids and of aziridine. In this paper we examine polycondensation reactions of formaldehyde with urea and with phenols—reactions that are of potential prebiotic importance.

Materials and Methods

Formaldehyde, 37.5% aqueous solution, with 11.6% of methanol as stabilizer, analytical reagent, was purchased from Mallinckrodt; 1,4-hydroquinone, 99+%, 4-hydroxybenzenesulfonic acid, sodium salt dihydrate, 98% (HBSA), 4-hydroxybenzoic acid, sodium salt, 99% (HBA), 2-hydroxybenzyl alcohol, 99% (HBzOH), and potassium cyanate, 97%, from Aldrich; phenol, redistilled, ultrapure, from ICN Biochemicals, Inc.; and urea, enzyme grade, from Life Technologies, Inc.

NENSORB 20 nucleic acid purification cartridges were obtained from DuPont, NEN Research Products, and presiliconized polypropylene microcentrifuge tubes from National Scientific Supply Co., Inc.

Synthesis of Oligonucleotides. The octanucleotide T_8, its amino-terminated analogue, T_8~NH_2 (the amino group is attached to the 3′-terminus of T_8 by an -O-PO_2^--O-CH_2-CH(OH)-CH_2-linker), and their [^{32}P]-labeled derivatives were prepared and purified as previously reported (Kolb and Orgel 1994).

pT_8~urea, a urea derivative of pT_8~NH_2, was prepared by a modification of a published procedure for the synthesis of substituted ureas from primary amines and cyanic acid (Hickinbottom 1962). 10 µl of pT_8~NH_2 (e.g., 18,400,000 cpm) was mixed with 25 µl 1.0 M KOCN and 25 µl of 0.1 M acetic acid and let stand at room temperature for 26 h; 25 µl of 0.1 M NH_4OH was then added and the mixture was passed through a NENSORB column. The eluted product, pT_8~urea, (13,500,000 cpm; 73% yield) was shown to be pure by gel electrophoresis. pT_8~urea moves slightly faster than the pT_8~NH_2 on a denaturing polyacrylamide gel.

Copolymerization of Formaldehyde with Urea, Phenol, and Substituted Phenols in the Presence of pT_8~Urea. In preliminary experiments we allowed formaldehyde to copolymerize with urea, phenol, or

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Abbreviations: HBA, 4-hydroxybenzoic acid, Na^+ salt; HBzOH, 2-hydroxybenzyl alcohol; HBSA, 4-hydroxybenzenesulfonic acid, Na^+ salt

a substituted phenol in the presence of [^{32}P]-labeled pT_8~urea at various pHs and temperatures. We found that the polymerizations were conveniently studied using concentrations of each monomer of about 0.25 M. Reactions were carried out at 67°C for 6 h at pHs in the range of 3.6–6.7. Lowering of pH to 2.9 resulted in a partial destruction of the oligonucleotide probe, as did prolonged heating.

The reactions were run in presiliconized RNase-free microcentrifuge tubes. Each reaction was run simultaneously on a small scale in the presence of [^{32}P]-labeled pT_8~urea in a 0.65-ml tube and on a larger scale omitting the [^{32}P]-labeled target in a 1.7-ml tube. The contents of the larger tube were inspected visually for the presence of precipitates or oils. The pH was measured in the large tube before and at various times during the reaction.

In a typical experiment a solution of pT_8~urea (5 µl; min. 3,000 cpm/µl) was mixed with 2.5 µl of a solution of formaldehyde (1.0 M) and 2.5 µl of a solution of urea (1.0 M), phenol (0.7 M; saturated solution) or one of the substituted phenols (1.0 M except for the less-soluble compounds: 0.66 M for HBSA and 0.50 M for hydroquinone); 2.5 µl of water was added in the reaction of pT_8~urea with formaldehyde alone. The tube containing the reaction mixture was placed in the heating block and kept at 67°C for 6 h; 5 µl of methanol was then added to the samples containing phenol or one of its derivatives. The samples were analyzed by gel electrophoresis/autoradiography. All samples were heated for 5 min at 67°C prior to application to the gel.

The pH values of the reaction mixtures at the beginning of the reaction were formaldehyde, 4.2; formaldehyde and urea, 4.5; formaldehyde and phenol, 3.8; formaldehyde and HBA, 6.7; formaldehyde and HBzOH, 3.8; formaldehyde and HBSA, 3.9; formaldehyde and hydroquinone, 3.6. At the end of the reaction time the pHs had not changed by more than 0.1 pH units in most cases. However, in the reaction with urea the pH increased by 1.2 units, and in the reaction with hydroquinone the pH decreased by 0.3 units. We found that buffering the formaldehyde/urea reaction mixture at pH 4.5 with 0.3 M sodium acetate did not change the course of the polymerization.

Results and Discussion

The oligonucleotide probe pT_8~urea was synthesized from pT_8~NH_2 (Kolb and Orgel 1994) in 73% yield by a modification of a known procedure for the synthesis of monoalkyl ureas (Hickinbottom 1962). pT_8~urea reacts with formaldehyde to give the methylol derivative which moves slightly slower than the pT_8~urea on a denaturing polyacrylamide gel (Fig. 1a). This reaction is analogous to the well-known reactions of urea and amides with formaldehyde, which give methylol derivatives. These methylolations have been reported to occur at pHs 2–12, with favorable, pH-independent equilibrium constants K ($K \approx 27$ and $K \approx 22$ for urea and amides, respectively) (Crowe and Lynch 1950; de Jong and de Jonge 1952a,b; Landqvist 1955a–d; Ugelstad and de Jonce 1957; Vail et al. 1962).

When pT_8~urea reacted with a formaldehyde-urea mixture, several bands were observed in the gel electrophoretogram (Fig. 1b). The fastest-moving band was assigned to the methylol derivative (Fig. 1a). The other bands represent condensation products shown in Fig. 2. After more extensive reaction, individual bands are not resolved. Under these conditions, visual inspection of the reaction vessel reveals progressive precipitation. This is in agreement with the literature reports that methylol

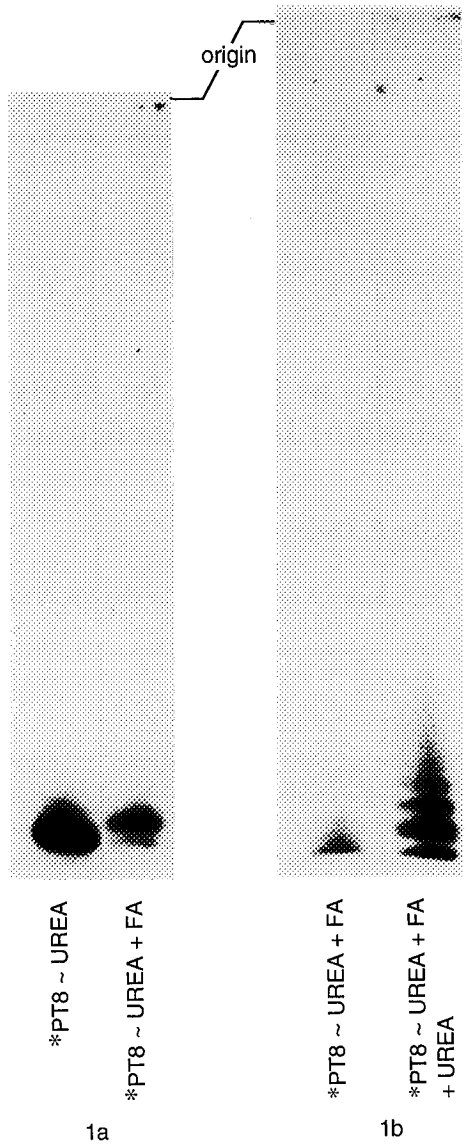

Fig. 1. **a** Gel electrophoretogram of *pT_8~urea (left) and its methylol derivative obtained by heating *pT_8~urea with formaldehyde (FA) (right). **b** Gel electrophoretogram of the methylol derivative of *pT_8~urea (left, compare Fig. 1**a**) and the condensation products obtained by heating *pT_8~urea with formaldehyde (FA) and urea (U) (right).

ureas condense rapidly under acidic conditions to give linear methylene ureas, which eventually give insoluble condensation products (Stevens 1990a; Walker 1964a).

We observed that the pH increased with time in our experiments. This is probably due to the decomposition of urea to give ammonium salts, a process that has been observed before (de Jong 1950; Smythe 1951). However, the inclusion of a buffer in the reaction mixture did not significantly effect the course of the reaction. The presence of methanol as a stabilizing agent in formaldehyde has been shown to slow down the polymerization because methanol reacts with formaldehyde to form a hemiacetal (Landqvist 1957). The overall influence of methanol in our case is believed to be small.

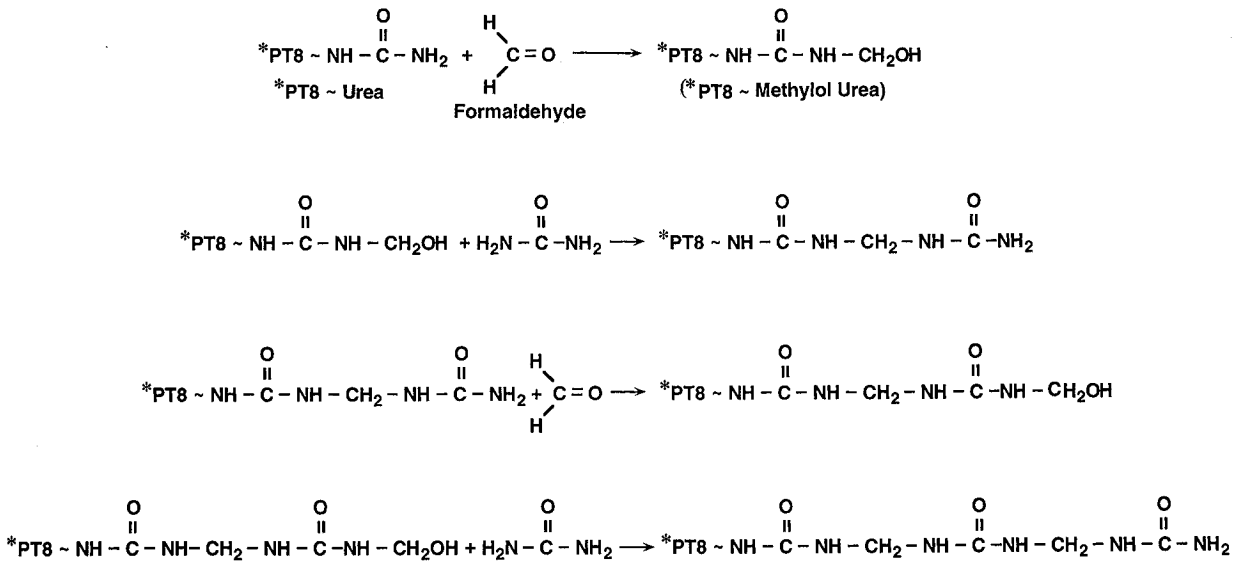

Fig. 2. Initial products of urea-formaldehyde condensation in the presence of *pT_8~urea.

The urea-formaldehyde reaction described above cannot be compared with the reactions that have been described in the literature. The latter typically describe commercially useful reactions in which water-soluble long urea-formaldehyde polymers are formed under *basic* conditions, and are subsequently crosslinked or aggregated under *acidic* conditions to give insoluble polymers.

The reactions involving formaldehyde and phenol gave oils which could not be completely dissolved even after addition of methanol and heating. Up to 60% of the material remained undissolved and/or stuck to the tube and pipette. The soluble material analyzed by gel electrophoresis consists of a mixture of adducts of short oligomers, as evidenced by the appearance of several bands on the film (Fig. 3). These bands are close together. A heavy background suggests the existence of unresolved products. The slowest-moving band is assigned to the methylol derivative of pT_8~urea (comparison gel not shown). This product reacts with phenol to give a methylene phenol derivative which undergoes further formaldehyde-phenol condensations. Figure 4 depicts potential products of the condensation containing one or two phenol residues. Under our pH conditions one would expect predominance of *ortho* substitution (Knop and Pilato 1985; Stevens 1990b).

The pH at which we ran our reaction, ca. 4, is the pH at which the methylolation of phenol with formaldehyde is the slowest (in the pH range of 1–11 [de Jong and de Jonge 1953]). However, as soon as methylol phenol is formed, it reacts further (at acidic pH) to form condensed products (Walker 1964b). In our case the decreasing solubility of oligomers which causes their oiling out of solution would terminate the reaction.

The substituted phenols that we studied all reacted with formaldehyde to some extent (gels not shown). The

Fig. 3. Gel electrophoretogram of the products obtained by heating *pT_8~urea with phenol and formaldehyde (FA).

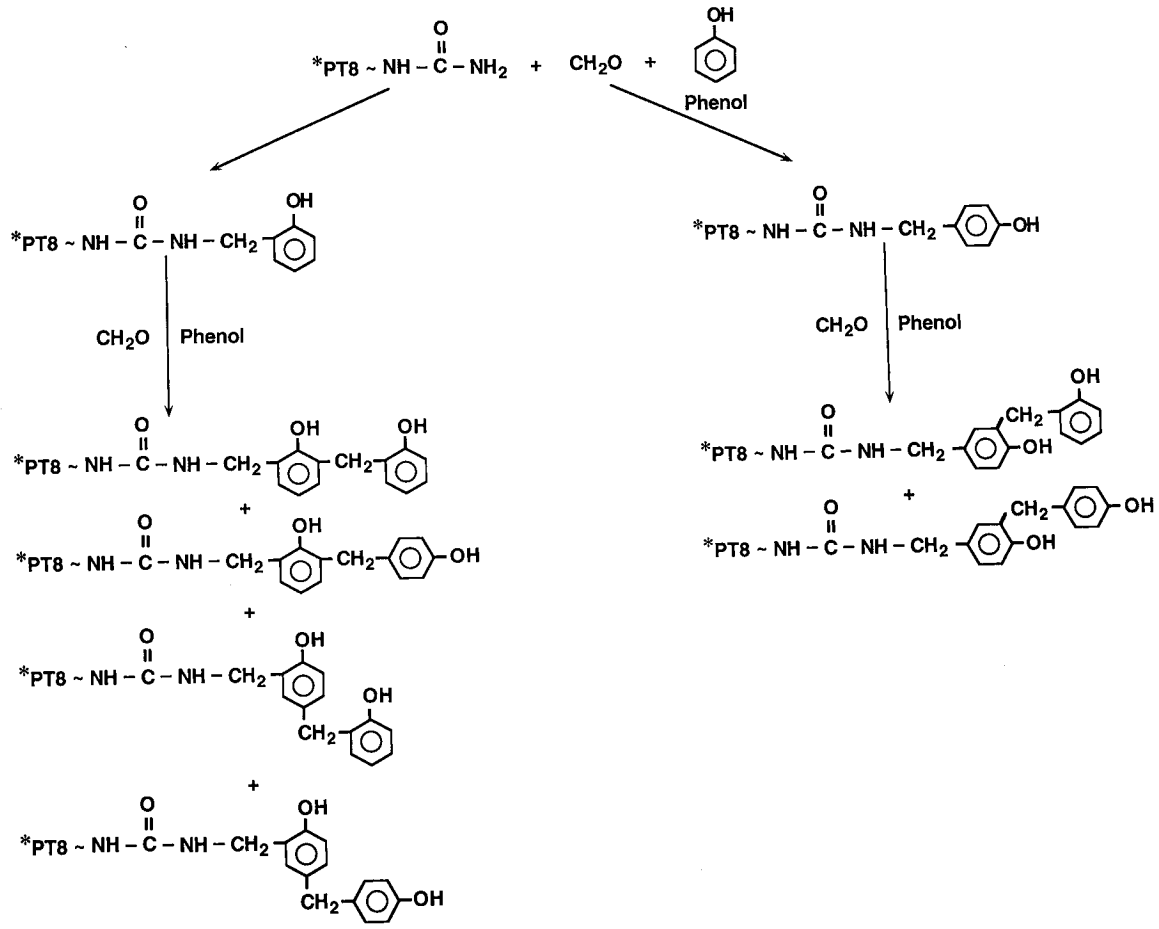

Fig. 4. Initial products of phenol-formaldehyde condensation in the presence of *pT_8~urea.

uncharged phenols, HBzOH and hydroquinone, reacted less extensively than phenol itself in agreement with previous reports (Walker 1964c). The extent of polycondensations of the two negatively charged phenols, HBA and HBSA, is difficult to compare with that for phenol because individual bands are poorly resolved on the gels. Presumably, the effect on the electrophoretic mobilities of the additional negative charge resulting from each additional phenol residue partially cancels the effect of the added mass. Under the conditions studied, the condensation reaction with HBSA was much more extensive than that with HBA.

The products of the reaction of HBSA did not give narrow, resolved bands on gel electrophoretograms (Fig. 5, lane 1). We showed that this was not due to self-aggregation by isolating samples from the top, middle, and bottom of the broad product band and determining that they retained their original positions on the gel when subjected again to electrophoresis (Fig. 5, lanes 2–4). If, as suggested above, the separation between the bands due to successive oligomers is small, the breadth of the product band suggests that long oligomers are present. We believe that HBSA gives longer oligomeric products than the other phenols, because oligomers of HBSA do not precipitate.

The results obtained in this work are in general agreement with those reported in the literature for those systems that have previously been studied. The advantage of our method is largely in speed and flexibility. Using conventional chromatographic separations of oligomeric products it is often necessary to develop a new chromatographic procedure and a new detection method when the nature of the monomer copolymerized with formaldehyde is changed—detection can be troublesome if the monomer does not absorb in the visible or ultraviolet. Using gel electrophoresis in association with [^{32}P]-labeling, the nature of the monomer can be changed—for example, from urea to phenol—without any need to change the separation or detection methods.

Urea and formaldehyde are amongst the most plausibly prebiotic molecules (Miller and Orgel 1974a), while phenols might possibly have formed on primitive Earth. Furthermore, many amino acids and amides also form copolymers with formaldehyde. It is unlikely that concentrations as high as those used in our experiments could have accumulated in the primitive ocean. However, evaporation of more dilute solutions could have resulted in very concentrated aqueous solutions (Miller and Orgel 1974b) in which extensive polymerization is possible. Formaldehyde copolymers, therefore, should be

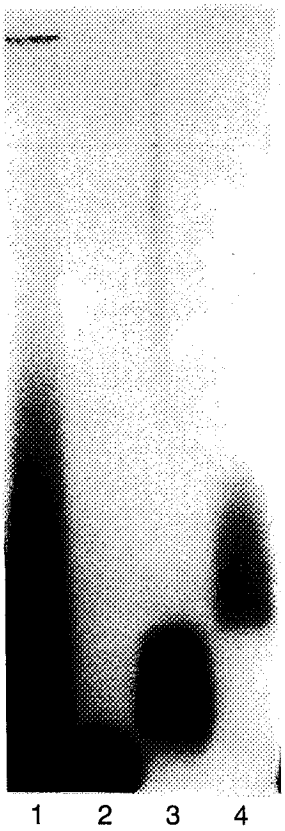

Fig. 5. Gel electrophoretogram of the products obtained by heating $*pT_8$–urea with HBSA (*lane 1*) and the lower (*lane 2*), middle (*lane 3*), and upper (*lane 4*) sections of *lane 1*, reelectrophoresed after elution from the original gel.

included in the rather short list of prebiotically plausible polymers that may have formed the basis for an early replication system.

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